

Sensitivity of digoxigenin and biotin labelled probes for detection of human papillomavirus by in situ hybridisation

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Abstract

The sensitivity of digoxigenin and biotin labelled DNA probes for the detection of human papillomavirus (HPV) by dot blotting and in situ hybridisation was compared in tissues from cervical, laryngeal, and anogenital neoplasia. Probes were either labelled with digoxigenin by the random primer technique and detected with anti-digoxigenin antibody, or labelled with biotin by nick translation and detected with streptavidin, both methods having a common final visualisation procedure using alkaline phosphatase. Digoxigenin labelled probes proved two to 10-fold more sensitive by quantitative dot blotting and four-fold more sensitive in detecting HPV 16 DNA in a series of 31 anal carcinomas, compared with biotinylated probes. The digoxigenin method also produced less non-specific background staining of tissue sections than biotin labelled probes.

It is concluded that digoxigenin DNA labelling and detection provides a simple, reliable, and efficient alternative to the use of biotin or radioactive isotopes for the detection of HPV DNA by in situ hybridisation. Digoxigenin labelled probes also offer the possibility of double labelling in situ hybridisation procedures when used with biotin labelled probes to provide simultaneous identification of different DNA sequences.

Human papillomavirus (HPV) infection has been strongly implicated in the development of cervical, laryngeal, and anogenital neoplasia, with low grade tumours usually associated with HPV types 6b and 11, and high grade with HPV types 16 and 18.¹⁻³ Although several methods are available for HPV detection,⁴⁻⁹ in situ hybridisation offers the advantage of cellular localisation of viral DNA within tissue sections and it can also be used with routinely collected paraffin wax embedded material, permitting retrospective investigations.

Of the variety of in situ hybridisation techniques available, radioactively labelled probes, although highly sensitive, have the disadvantage of safety hazards associated with ionising radiation and long autoradiography exposure times of up to 30 days.¹⁰ The use of biotin as a non-radioactive label has proved popular^{11,12} despite the disadvantage of non-specific bind-

ing of streptavidin to endogenous sites within tissues.^{13,14} As a putatively more sensitive alternative, digoxigenin, a derivative of the cardiac glycoside digoxin, has recently been used to label DNA probes for the successful identification of HPV in HeLa and SiHa cells.¹⁵

Methods

TISSUE PREPARATION

Procedures were adapted from the method of Lewis *et al.*¹² Paraffin wax sections (5 μ m) were prepared from routinely processed paraffin wax blocks and placed on 3-aminopropyltriethoxysilane (TESPA; Sigma) coated slides¹⁶ and incubated at 42°C for three days before dewaxing in xylene at 37°C for 30 minutes and xylene at room temperature for 10 minutes. Tissue sections were hydrated through absolute ethanol twice for five minutes, 95%, 85%, 70% and 50% (v/v) ethanol for two minutes each, and phosphate buffered saline (PBS) for five minutes. They were then immersed in 0.02M hydrochloric acid for 10 minutes, washed in PBS twice for five minutes, placed in 0.1% (v/v) Triton X-100 in PBS for one and a half minutes, washed in PBS twice for five minutes, and digested with 250 μ g/ml proteinase K (Gibco-BRL) in 50 mM TRIS (pH 7.6), 5 mM EDTA for 10 minutes at 37°C. Further digestion was prevented by washing the slides twice in PBS containing 2 mg/ml of glycine for five minutes, and endogenous alkaline phosphatase activity was inhibited by immersing in 20% (v/v) acetic acid at 4°C for 15 seconds. This was followed by two 10 minute washes in PBS, post-fixation in 4% (w/v) paraformaldehyde in PBS for five minutes, two five minute washes in PBS, and dehydration through graded alcohols, as above, to absolute ethanol where the slides were stored awaiting hybridisation.

For the detection of highly repetitive sequences, human cells from male buccal mucosa were smeared on to glass microscope slides and allowed to air dry for one minute and treated as described below for the detection of the Y chromosome.¹⁷

Frozen sections (5 μ m) were placed on TESPAs coated microscope slides and fixed in 4% paraformaldehyde in PBS for 16-24 hours, washed twice in 0.1M TRIS (pH 7.4) for five minutes, immersed twice in 0.25% (v/v) Triton X-100, 0.25% (v/v) Nonidet P40 in 0.1M TRIS for five minutes, and washed twice in 0.1M TRIS for five minutes. Slides

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were subsequently incubated at 37°C with 100 µg/ml of proteinase K in 50 mM TRIS, 5 mM EDTA for 10 minutes, washed in 0.1M TRIS containing 2 mg/ml glycine twice for five minutes and immersed in 20% (v/v) acetic acid at 4°C for 15 seconds before being washed twice in 0.1M TRIS for five minutes.

Slides for subsequent hybridisation with biotin-labelled probes had endogenous avidin/biotin binding sites blocked using the recommended protocol described by the supplier (Vector Laboratories). Briefly, the slides were placed in a humidified box and sections covered with avidin for 20 minutes before washing twice in 0.1M TRIS for five minutes. The slides were returned to the humidified box and sections covered with biotin for 20 minutes and washed twice in 0.1M TRIS for five minutes. All slides were then immersed in 2 × SSC (0.3M sodium chloride, 30 mM sodium citrate at pH 7.0) for 10 minutes and 2 × SSC, 50% deionised formamide for 60 minutes.

PREPARATION OF DNA PROBES

Plasmids containing HPV 11 or 16 cloned into pBR322 (donated by Dr L Gissmann, Heidelberg) or human Y chromosome sequences cloned into pBR328 (pHY2.1) (from Dr HJ Cooke, Edinburgh) were labelled with either biotin-11-dUTP by nick translation¹⁸ or digoxigenin-11-dUTP by random primer labelling using commercially available kits (Gibco-BRL, Boehringer) following the recommended protocols.¹⁹⁻²¹ DNA (1 µg) was labelled with biotin. With digoxigenin either 1 µg (dot blots) or 0.2 µg (in situ hybridisation and dot blots) were labelled. In some experiments DNA was labelled with digoxigenin by nick translation. Before random primer labelling the DNA was linearised with the restriction enzymes *Bam* *H*1 (HPV 11 and 16) or *Eco* *R*1 and *Pvu* *II* (pHY2.1). Unincorporated nucleotides were removed using GeneClean (Stratagene). For in situ hybridisation, DNA probes were prepared at a concentration of 200 ng/ml (biotin) or 140 ng/ml (digoxigenin) in hybridisation buffer containing 2 × SSC, 5% (w/v) dextran sulphate, 50% deionised formamide, and 0.2% (w/v) low fat skimmed milk. Labelled DNA for dot blot hybridisation was prepared at a concentration of 40 ng/ml in 5 × SSC, 0.1% (w/v) N-lauryl sarcosine, 0.02% (w/v) sodium dodecyl sulphate (SDS), 0.5% (w/v) blocking reagent (phial 11, Boehringer).

DOT BLOT PREPARATION AND HYBRIDISATION

Aliquots (2 µl) of heat denatured HPV or salmon sperm DNA were serially diluted in 10 mM TRIS (pH 7.4) 1 mM EDTA (TE), spotted on to nitrocellulose filter paper, and baked under vacuum at 80°C for two hours. Filters were pre-hybridised in the above buffer at 65°C for six hours and hybridised in 2.5 ml of fresh buffer containing 100 ng of heat denatured HPV 16 probe for 16 hours at 65°C. Filters were washed twice in 2 × SSC, 0.1% (w/v) SDS for five minutes at room temperature, followed by 0.1 × SSC, 0.1%

(w/v) SDS twice for 15 minutes at 65°C. In some experiments labelled probe was spotted directly on to nitrocellulose and baked under vacuum at 80°C for two hours.

IN SITU HYBRIDISATION

Hybridisation mixture (50 µl) containing the labelled DNA was placed on the prepared sections, covered with Gelbond (ICN), and the edges sealed with nail varnish. Probe and cellular DNA were denatured together by heating to 90°C for 10 minutes and the slides transferred to a humidified box at 42°C for 16 hours. After removal of the Gelbond the slides were washed at high stringency in 2 × SSC at room temperature for 10 minutes, 2 × SSC at 60°C for 20 minutes, 0.2 × SSC at room temperature for 10 minutes, and 0.2 × SSC at 42°C for 20 minutes.

DETECTION OF HYBRIDISED PROBE

Both the slides and the dot blots were processed at room temperature using the commercially available detection kits for use with biotin (Gibco-BRL) or digoxigenin (Boehringer), with slight modifications to the recommended protocols.

After washing for five minutes in buffer 1, containing either 0.1M TRIS (pH 7.5), 0.1M NaCl, 2 mM MgCl₂, 0.05% (v/v) Triton X-100 (biotin) or 0.1M TRIS pH 7.5, 0.15M NaCl (digoxigenin) the slides were immersed in buffer 1 containing 3% (w/v) bovine serum albumin (biotin) or 20% sheep serum (digoxigenin) for 30 minutes. Slides were then incubated in a humidified box with buffer 1 containing either 2 µg/ml of streptavidin (biotin) or a 1 in 5000 dilution of anti-digoxigenin antibody for 20 minutes before being washed in buffer 1, twice for 20 minutes. Biotin slides were returned to the humidified box, covered in buffer 1 containing 1 µg/ml of biotin-polyalkaline phosphatase for 20 minutes, before washing in buffer 1, twice for 20 minutes. All slides were then placed in buffer 2 containing 0.1M TRIS (pH 9.5), 0.1M NaCl, 5 mM MgCl₂ for 60 minutes to raise the pH. Visualisation reagent containing buffer 2, nitro-blue tetrazolium salt (0.33 mg/ml), and 5-bromo-4-chloro-3-indolyl phosphate (0.17 mg/ml) was then applied. Colour development was allowed to proceed in the dark for up to three hours (biotin) or up to 16 hours (digoxigenin) and the reaction stopped by immersion in 20 mM TRIS (pH 7.5), 5 mM EDTA before mounting in aquamount (BDH).

Dot blots were treated as above with all reagent applications in sealed polythene bags and wash steps undertaken in plastic boxes.

Results

DOT BLOT ANALYSIS: INCORPORATION OF DIGOXIGENIN AND BIOTIN LABELLED NUCLEOTIDES INTO DNA PROBES

The quantity of labelled nucleotide incorporated into the probe was dependent of both the amount of template DNA used and the labelling method. A minimum of 0.1 pg of digoxigenin labelled DNA probe (following

random primer labelling of 0.2 µg HPV 16 DNA) was visualised following direct spotting of labelled probe DNA on to a nitrocellulose filter (fig 1; lane A), a minimum of 0.5 pg was visualised if the labelling reaction required 1 µg of DNA (fig 1; lane B). This result was repeatedly confirmed and suggests that smaller quantities of DNA incorporated proportionately more label.

Probe DNA (1 µg) labelled by nick translation with either biotin (fig 1; lane C) or digoxigenin (fig 1; lane D) resulted in a minimum detection limit of 1 pg. This was modified to 0.5 pg when only 0.2 µg of DNA was labelled with digoxigenin by nick translation (fig 1; lane E). Thus by direct visualisation of labelled DNA, probes prepared by random primer labelling showed a two to five-fold increase in label incorporation compared with probes prepared by nick translation.

HPV 16 DNA probes, labelled as above with

either digoxigenin by the random primer technique (0.2 µg DNA) or biotin by nick translation (1 µg DNA), were hybridised to HPV 16 target DNA immobilised on nitrocellulose filters (fig 2). Digoxigenin labelled probes detected a minimum of 2.5 pg of target DNA (lane A) compared with a minimum of 25 pg for biotin labelled probes (lane C), indicating a 10-fold difference in sensitivity. Neither probe hybridised to salmon sperm DNA used as a negative control (lanes B and D).

IN SITU HYBRIDISATION

Digoxigenin and biotin labelled probes were used in parallel to detect the presence of HPV DNA in cell smears or tissue sections by in situ hybridisation. Positive hybridisation was identified as purple-blue granular deposits localised in cell nuclei.

Heavily stained nuclei were observed after hybridisation with digoxigenin labelled HPV 16 DNA in paraffin wax sections of an anal carcinoma (fig 3A) and in frozen sections of a lesion containing cervical intraepithelial neoplasia (CIN III) (fig 3C). Under identical conditions, using adjacent sections, DNA probes labelled with biotin failed to produce nuclear staining in the anal carcinoma (fig 3B) and produced much reduced staining in the frozen section of CIN III (fig 3D). The detection of highly repetitive sequences, as seen in the human Y chromosome, resulted in similar intensely staining spots within the nuclei of cell smears with both digoxigenin (fig 3E) and biotin (fig 3F) labelled pHY 2.1 probe DNA.

A series of 31 invasive anal carcinomas was investigated for the presence of HPV 16 DNA by in situ hybridisation using both digoxigenin labelled and biotinylated probes applied to adjacent paraffin wax sections. Twelve (39%) cases were positive using digoxigenin labelled probes; only three (10%) were positive with biotinylated probes, showing a significant difference ($p < 0.01$; χ^2 test) between the two methods using routinely processed tumour tissue. The three cases identified using biotinylated probes were all positive with digoxigenin labelled probes. In 13 of 31 cases more than one block of tumour was examined; six of these cases contained HPV 16 DNA using digoxigenin labelled probes, five were positive in all blocks tested, with a single exception in which one block out of two was negative.

Considerable non-specific background staining was observed in the paraffin wax sections of anal carcinoma hybridised with the biotinylated probes after three hours of colour development (fig 3B). By contrast, digoxigenin labelled probes produced little background even after 16 hours of colour development (fig 3A). Hybridisation of digoxigenin random primer labelled HPV 11 DNA probes to paraffin wax sections of a laryngeal papilloma was clearly observed within three hours of colour development (fig 4A), but a much stronger signal was observed when this was left for 16 hours, with little background staining (fig 4B).

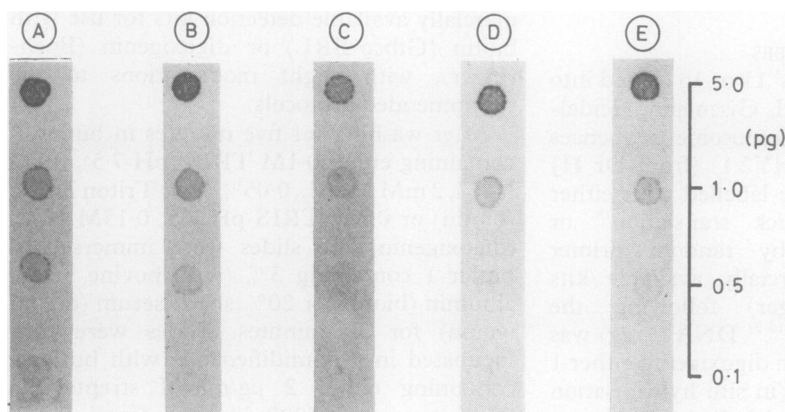


Figure 1 Dot blot comparison of HPV probe DNA labelled by nick translation and the random primer method. Labelled HPV 16 DNA was directly immobilised on to nitrocellulose after serial dilution, with deposition of coloured reaction product over 16 hours. The minimum detection limits are 0.1 pg for 0.2 µg (lane A) and 0.5 pg for 1 µg (lane B) of DNA labelled with digoxigenin by the random primer technique. There was a similar detection limit of 1 pg when 1 µg of DNA was labelled with biotin (lane C) or digoxigenin (lane D) by nick translation. Digoxigenin labelling of 0.2 µg of DNA by nick translation produced a detection limit of 0.5 pg (lane E).

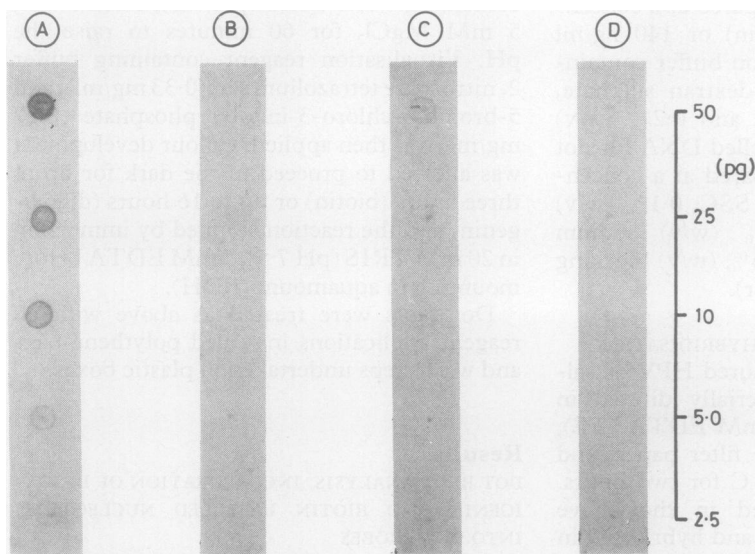


Figure 2 Dot blot hybridisation of labelled HPV 16 DNA to homologous target (lanes A and C) or control salmon sperm DNA (lanes B and D). The probes were labelled with either digoxigenin using the random primer technique (lanes A and B) or with biotin by nick translation (lanes C and D). No hybridisation is observed between salmon sperm DNA and either HPV 16 DNA probe (lanes B and D).

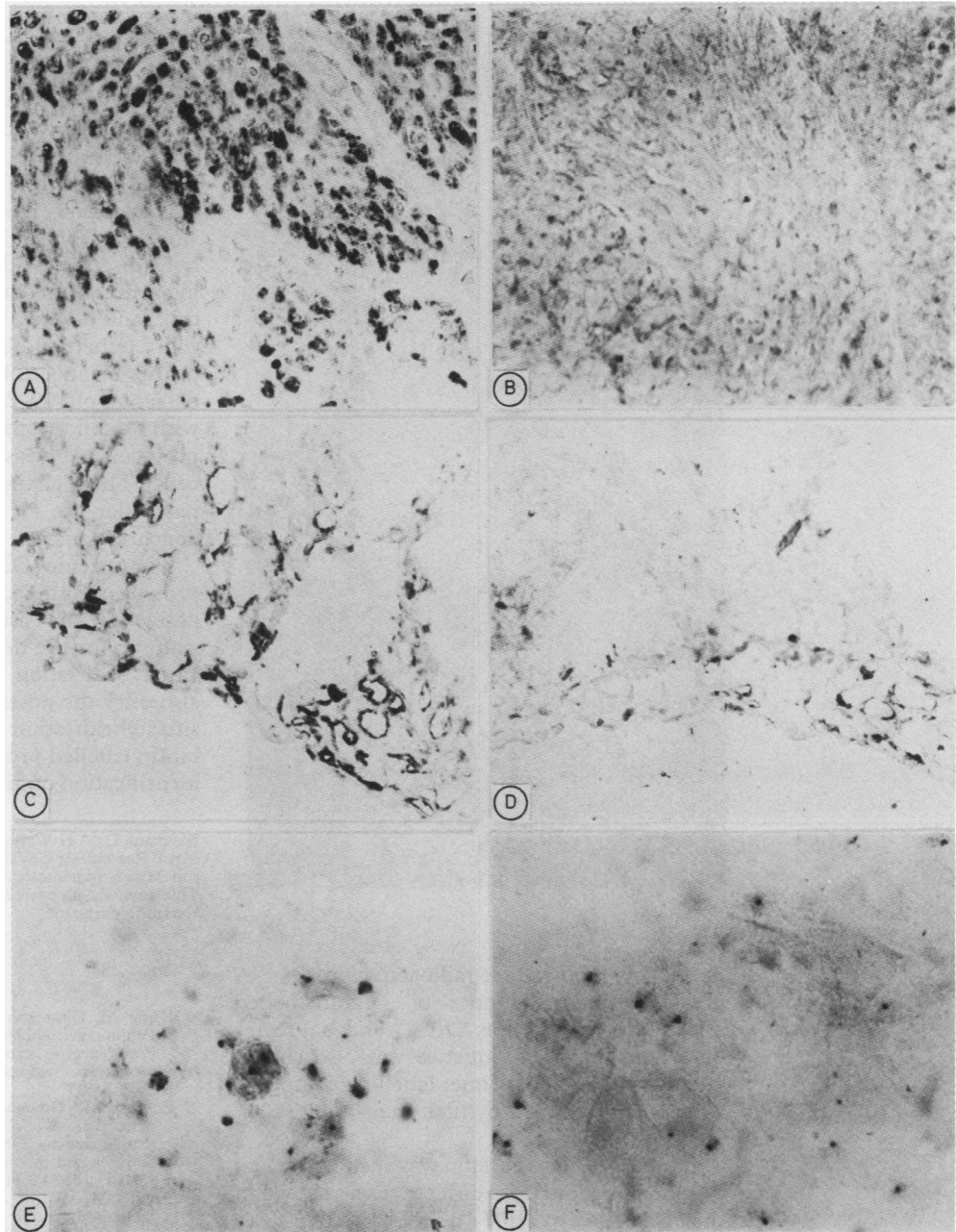


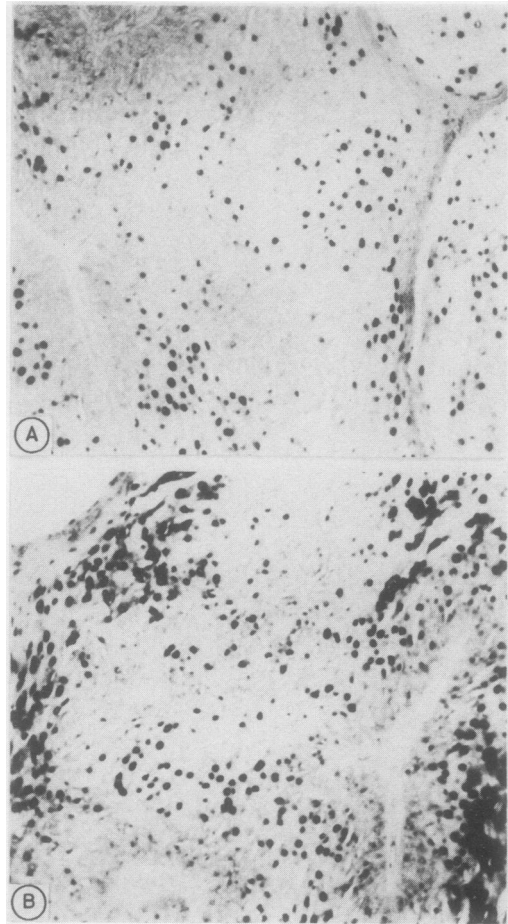
Figure 3 In situ hybridisation of labelled HPV 16 DNA to adjacent paraffin wax sections of an anal carcinoma (A and B) and frozen sections of cervical intraepithelial neoplasia (C and D). Positive staining can be clearly identified in cell nuclei after hybridisation with random primer/digoxigenin labelled probe (A and C). The use of probes labelled with biotin by nick translation did not produce specific nuclear staining in paraffin wax sections of the anal carcinoma (B) and gave a diminished intensity of staining in frozen sections of cervix (D). In situ hybridisation using pHY2-1 probes produced an intense staining spot within the nuclei from cell smears with either random primer/digoxigenin (E) or biotin/nick translated (F) labelled DNA. The duration of colour development was three hours for all biotin probe hybridisations and either three hours (frozen sections and cell smears) or 16 hours (paraffin wax sections) for digoxigenin probe hybridisations.

Discussion

Labelling of HPV DNA with either digoxigenin by the random primer method or biotin by nick translation produces probes which have different sensitivities in detecting the presence of target HPV DNA both in dot blots and by in situ hybridisation. Although both procedures gave satisfactory results with the identification of highly repetitive sequences, such as those of the human Y chromosome,¹⁷ the increased sensitivity of the digoxigenin/random primer technique resulted in both quantitative and qualitative differences in the detection of HPV infection.

The quantity of labelled nucleotide incorporated into DNA probes clearly has a major influence on their sensitivity in detecting target sequences. Using direct detection of digoxigenin labelled DNA we have shown two to five-fold greater DNA labelling by the random primer method than by nick translation. Furthermore, random primer labelling of quantities of less than 1 µg of DNA apparently results in a proportionately greater incorporation of digoxigenin, presumably with a concomitant reduction in the percentage of non-labelled DNA able to compete in the hybridisation reaction. These findings are comparable with those

Figure 4 *In situ* hybridisation of random primer/digoxigenin labelled HPV 11 DNA to paraffin wax sections of a laryngeal papilloma after colour development for three hours (A) or 16 hours (B).



previously reported for radioactive probes.²¹ A greater incorporation of radiolabelled nucleotide into probes (70%) and a greater detection of target sequence was also shown when using random primer labelling compared with probes labelled by nick translation (10% incorporation).

When applied to paraffin wax sections of anal carcinomas a four-fold difference was shown in the proportion of HPV 16 DNA positive cases identified. The digoxigenin labelled probes with an anti-hapten antibody detection system identified 39% positive reactions from 31 cases, compared with only 10% for biotinylated probes with streptavidin detection. This is a substantial difference in sensitivity between these two methods using tumour blocks obtained in routine practice.

Using biotinylated probes, problems may also arise due to the presence of endogenous streptavidin binding sites within tissue sections, especially in frozen tissues.^{13,14} The non-specific background staining observed in the paraffin wax section of anal carcinoma masked the presence of hybridised biotinylated HPV DNA in the carcinoma cells. Using digoxigenin labelled probes, foci of cells containing HPV were clearly identified with little non-specific background, even after 16 hours of colour development. The superior results obtained with the digoxigenin method are unlikely to be due to differences in label attachment, as both digoxigenin and biotin are conjugated to dUTP by an 11 atom linker arm. The differences more probably result from a combination of greater

incorporation of label into DNA by the random primer method, better detection of digoxigenin label within tissue sections by the anti-hapten antibody, and a lower non-specific background staining due to the lack of endogenous anti-digoxigenin antibody binding sites, permitting longer times for label detection.

Controversy has arisen regarding the sensitivity of biotin labelled probes for *in situ* hybridisation, with some investigators claiming a minimum detection of 10 HPV genomes a cell²² and others a minimum of 800 genomes a cell.²³ Other studies using digoxigenin labelled probes for *in situ* hybridisation have indicated detection of 100–200 HPV genomes a cell and, with modifications to the *in situ* hybridisation protocol, as few as one to two HPV genomes a cell.¹⁵ The digoxigenin method may thus be of particular use for the detection of low abundance sequences.

Digoxigenin DNA labelling and detection provides a simple, reliable, and efficient alternative to the use of biotin or radioactive isotopes for the detection of HPV DNA by *in situ* hybridisation. Digoxigenin labelled probes also offer the possibility of double labelling in *in situ* hybridisation procedures when used with biotin labelled probes to provide simultaneous identification of different DNA sequences.

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